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Research Article

STEM CELLS FOR HEALING INDUCED CUTANEOUS WOUNDS IN DOGS

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Abstract

Adipose tissue stem cells (ASCs), known as multipotent stem cells, are most commonly used in the clinical applications in recent years. Adipose tissues (AT) have the advantage in the harvesting, isolation, and expansion of ASCs, especially an abundant amount of stem cells. The aim of this study was to evaluate the efficacy of Adipose tissue stem cells (ASCs) in skin wound healing comparing to control group. Four 25 mm diameter full-thickness circular wounds were made in the back area under general anesthesia of 8 dogs then divided randomly in to two equal groups (control and stem cells groups). Wounds were grossly and histopathologically evaluated on days 7, 14, 21, and 28 for evaluation of the healing process. The results showed progressive reduction in the wound surface area with time in Treated groups (T1) compare to the control. The best result was showed in the Stem cells group (T1) compare to the Control group. Adipose tissue Stem Cells (ASCs) treated group had superior wound surface reduction and healing when compared to control groups.

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1. Introduction

Wound is the disruption of the cellular and anatomic continuity of a tissue (Abdulkareem *et al*., 2019). Cutaneous wound healing is a complex process involving the interplay of different cell types in the wounded tissue, including inflammatory cells, fibroblasts, keratinocytes, and endothelial cells **(**Donald and Zachary, 2007; Lee *et al*., 2011). Appropriate wound care is critical and wound care aims to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive for tissue repair and regeneration (Martins *et al*., 2011; Ahmed Rasool *et al*., 2013).

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The stem cell is defined by the capability of self-renewal and differentiation into several types of cells. According to the different sources of the cell, they can be divided into an Embryonic Stem Cell (ESC), somatic stem cell, and induced pluripotent stem cell (Lenoir, 2000; Takahashi and Yamanaka, 2006; Duan *et al*., 2011). In recent years, Adipose-Derived Stem Cells (ADSC) have emerged as a promising cell source for tissue engineering because the adipose tissue can be obtained in large quantities without causing discomfort to the patients and can enrich a lot of stem cells(Lindroos *et al*., 2001; Meliga *et al*., 2007; Mizuno *et al*., 2012).In addition, there is no ethical problem to get the adipose tissue for isolation of ADSCs. The aim of present

study to a comparison to the use of stem cells group and control group in the wound healing induced in the skin of the dogs.

2. Materials and Methods

The study was conducted in the College of Veterinary Medicine, University of Basrah, Iraq. Dogs were a study model. Eight healthy mongrel dogs, the age range between 1 - 1.5 years; weight ranges between 15 - 18 kg were used in this study. The animals were divided into two equal groups each group has 4 animals (1 treatment groups and 1 control group). Dogs were subjected to complete physical examination, including complete blood cell count and serum biochemistry analyses to evaluate the general health status of the animals before the inclusion in the study which drawn blood from the cephalic vein (Auda, Ahmed, 2019). Each dog was identified using collar ID and housed separately in cages and were provided with daily dry food and a libitum source of clean water.

Eight dogs divided in to two equal groups. One group treated (T1) and second one became control. The T1 treated with stem cells. Control group don't receive any materials. Adipose-derived stem cell preparation: Briefly, adipose tissue was obtained from subcutaneous, inguinal fat depots of dogs, using standard surgical procedures. At obtaining, adipose tissue was placed into a sterile 50 ml conical tube containing 15 ml of Phosphate - Buffered Saline (PBS) and brought quickly to stem cells laboratory.

Collected adipose tissue was washed three times with phosphate-buffered saline containing 100 IU/ml penicillin and 100 g/ml streptomycin, then chopping sample with scalpel blade size 15 and surgical forceps and digested for 1 hour at 37 °C with collagenase type IA. The enzymatic activity was inhibited with Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % Fetal Bovine Serum (FBS). Following centrifugation at 1200 x g for 5 min, the pellet was filtered through a 70 µm falcon cell strainer to remove debris, then incubated in DMEM containing 10 % FBS at 37 °C in a humidified atmosphere of 5 % CO_2. After 48 hrs, the culture was washed with PBS to remove non-adherent cells and incubate with a fresh medium, which will be changing every 48 hrs until cells reached 70 % to 80 % confluence. The cells were then repeatedly sub cultured under standard conditions (Lee *et al.*, 2017).

Wounds Induction

Experimental dogs were selected (n=4) for each group. Under strict aseptic conditions and general anesthesia using intramuscular injection mixture of Ketamine (10 mg/kg) (Al-Abaddi and Al-faris, 2021) and Xylazine (0.5 mg/kg) (Habeeb *et al*., 2021). On each dog, a total of four (2.5 cm in diameter) skin circular wounds were created aseptically in the back area using a customized skin punch biopsy kit (Figure -1).

a) *The first group (Treatment -T_1):* Group was treated 1×10^6 of MSCs diluted in 2 ml of PBS by injecting 1 ml at the center of the surface of the wound bed and their margins while the second ml was topically applied at the center of the same wounds. Then, covered with a bandage (Figure - 2).
b) *The control group:* Wounds of the control group were covered with a bandage and left without treatment as a control group (Alshehabat*et al*., 2020).

Clinical evaluation

The crated wounds were grossly evaluated once a day for clinical signs of wound healing and maturation (reconstruction or remodeling). Also clinically observed any complications and the wound site photographed immediately after wounding and then on days (7, 14, 21 and 28) post wound creation. The percentage wound contraction and total wound healing was calculated for each wound using the equation $[(A_0 - A_t)/A_0] \times 100 = \%]$ of wound closure (Agra *et al.*, 2013).

A_0 = The original wound area; A_t= Area of the wound at the time of biopsy

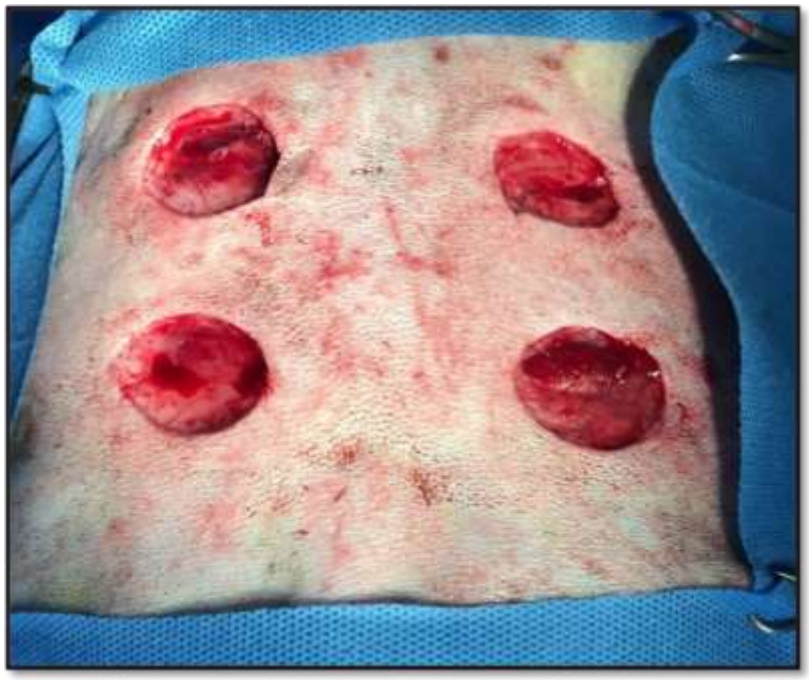

Figure - 1: Induction of skin circular wounds (25mm in diameter) were created aseptically in the back area

Figure - 2: Injecting the stem cells to the created wound

Histopathological evaluations

All biopsy samples of all groups were obtained under general anesthesia at days (7, 14, 21 and 28) after wounding using a circular excisional biopsy, and fixed in (10 %) neutral buffer formalin solution. The tissue specimens are processed in a tissue processor for the paraffin technique. Tissue sections were cut at 5 - 6 µm and stained with Hematoxylin and Eosin and Masson's trichrome stains (Hussein *et al*., 2011). The stained sections were blindly examined by a board-certified pathologist. A scoring system was developed to evaluate different histopathological parameters (Hananeh *et al*., 2015).

Statistical analysis

Data were collected, revised, coded, and entered into the Statistical Package for Social Science (IBM SPSS) version 20. The results obtained were statistically analyzed using t-test. All values were expressed as Mean ± Standard deviation. The level of significance was set at $p < 0.05$. Used (ANOVA) SPSS (2008).

3. Results

Macroscopic evaluation results

Macroscopical parameters were used to evaluate skin wound healing including percentage of wound contraction, wound clinical signs and complications. The results showed progressive reduction in the wound surface area with time in one treated groups (T_1) compare to the control one (Figures – 3 and 4) and the best result was showed in the stem cells group (T_1) compare to the control group.

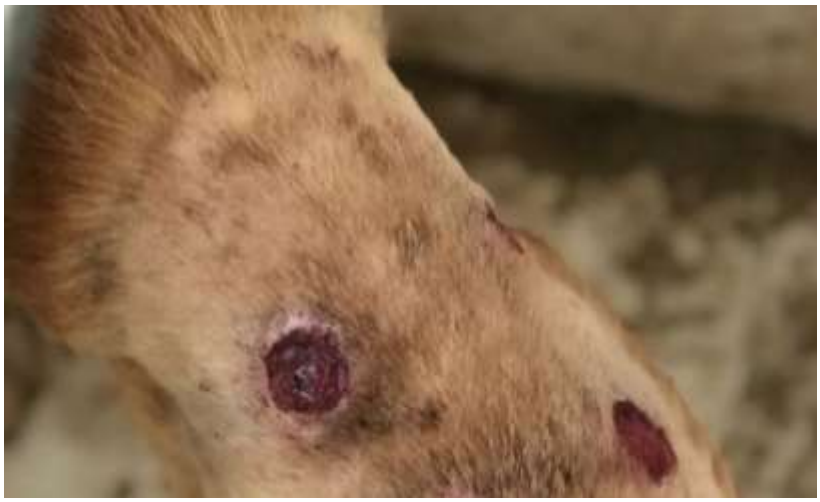

Figure – 3: Control group, four weeks post wound induction

Figure – 4: T1 group, four weeks after stem cells injection

Table - 1: Percentage of wound contraction among groups

Time/days	Control group	Stem cells group (T1)
7	12.5 % ± 0.57	42.00% ± 1.41
14	23 % ± 0.95	62.50% ± 1.73
21	31.66 % ± 0.95	69.00% ± 0.81
28	41 % ± 1.29	82.75% ± 1.89

The results of different percentages of wound contraction in showed higher mean percentage of wound contraction in group treated with Stem cells (T_1) (82.75 %) compare to Control group (41 %). Inflammation, transudate, redness and swelling were more noticeable in control group compare to treated group (Table - 1).

Microscopic Evaluation Results

Skin of control group showed sever inflammation in the site of wound after one week of wound induction (Figure - 5).

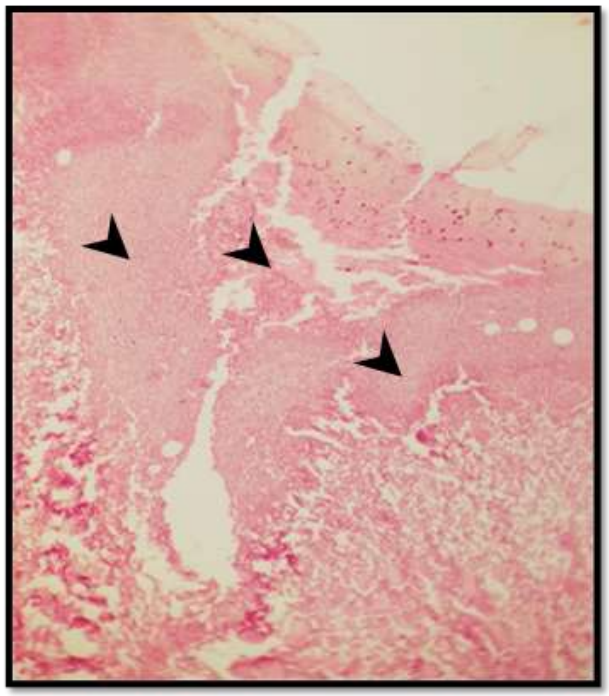

Figure - 5: Skin section of control group at 1st week post wound induction showed sever inflammation in the site of induced skin wound (black arrow head) H & E (125X)

While stem cells treated group showed moderate inflammation and development of newly generated blood vessels in the site of wound at the same period of the study (Figure - 6).

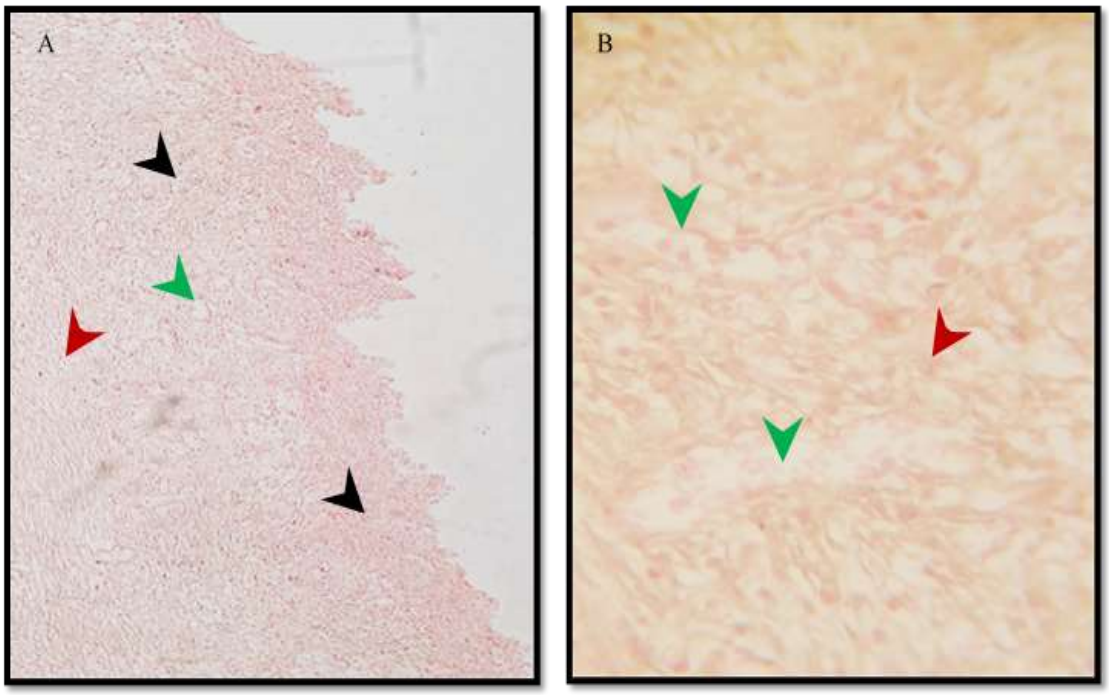

Figure - 6: Skin section of stem cells group at 1st week post treatment showed moderate inflammation in the site of induced skin wound (black arrow head). Development of granulation tissue in the dermal layer (red arrow head) with newly generated blood vessels (green arrow head) H & E A: (125X). B:(500X)

In the second week, still there were a massive inflammation in the site of the skin wound in the both control group (Figure - 7).

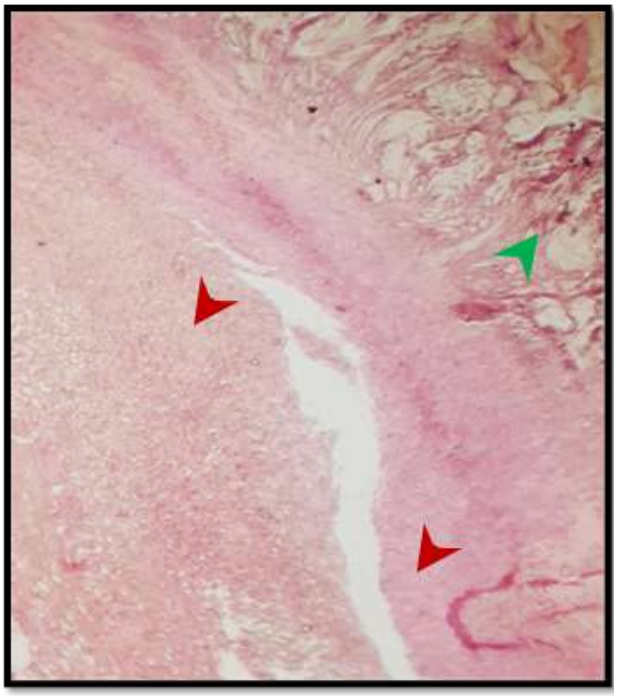

Figure - 7: Skin section of control group at 2nd week post wound induction showed sever inflammation in the site of induced skin wound (red arrow head) H & E (125X)

While the stem cells treated group showed moderate inflammation with initial evets of epithelial regeneration in the epidermal layer at the same period (Figure - 8).

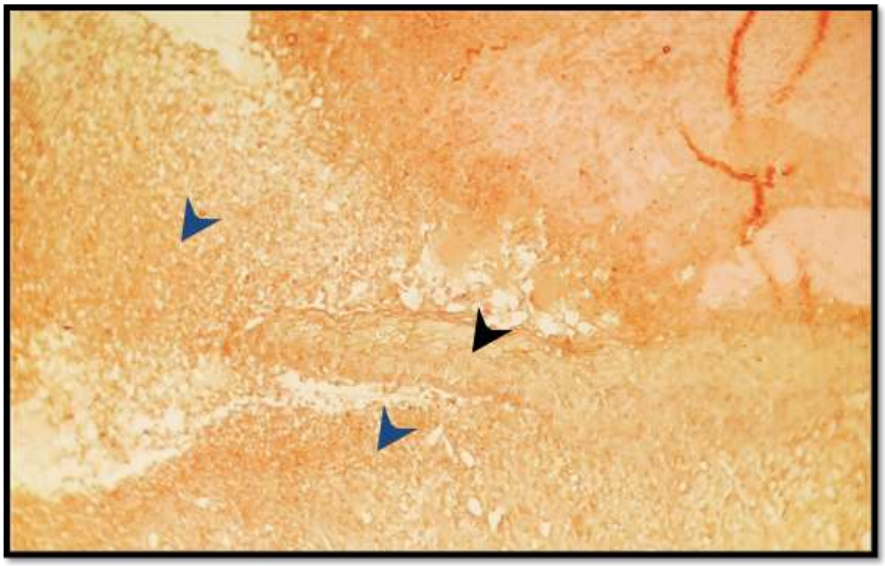

Figure - 8: Skin section of stem cells group at 2nd week post treatment showed moderate inflammation in the site of induced skin wound (blue arrow head). Regenerating skin epithelium in the epidermis (black arrow head) H & E(125X).

The events of sever inflammation was extended to the third week in the control group. Regarding the same period stem cells treated group showed marked re-epithelialization, with hyperplasia of the epithelial cells and complete regeneration of the epithelial basal layer, regenerating skin epithelium in the epidermis (Figure - 10).

Figure - 9: Skin section of control group at 3rd week post wound induction showed sever inflammation in the site of induced skin wound (black arrow head) H & E (125X)

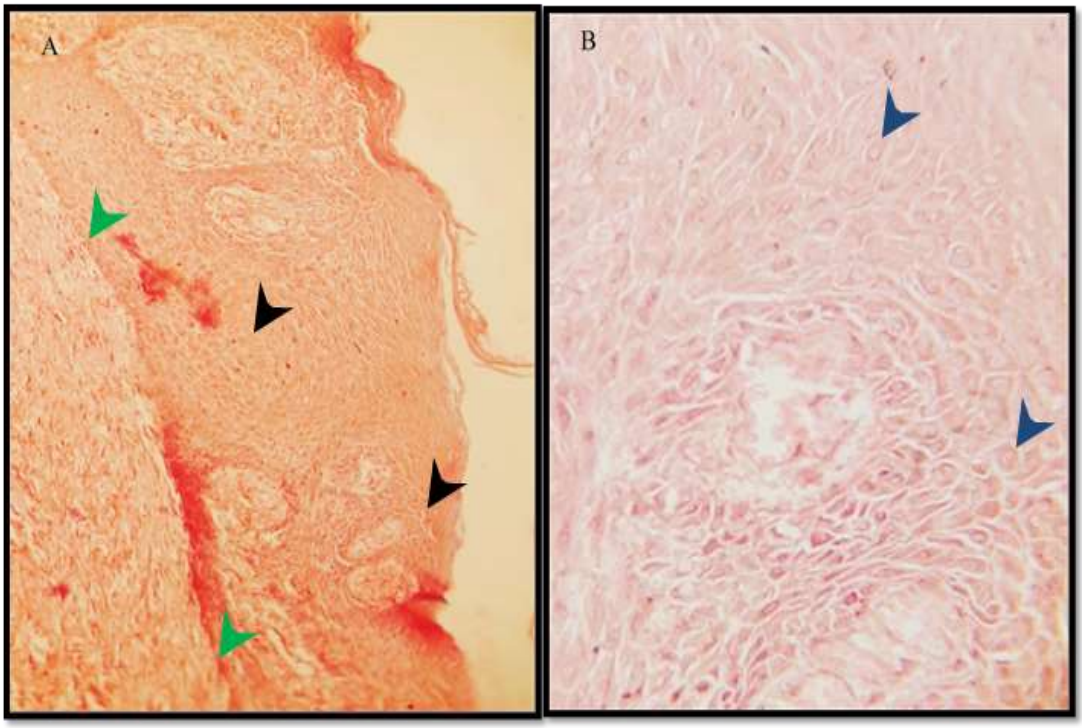

Figure - 10: Skin section of stem cells group at 3rd week post treatment showed marker re-epithelization in the site of skin wound (black arrow head) with hyperplasia of epithelial cells and complete regeneration of the epithelial basal layer (green arrow head), regeneration skin epithelial in the epidermis (blue arrow head) H & E A:(125X). B:(500 X)

Skin section of control animals at 4thweek showed newly generated vasculature in the dermal layer (Figure - 11).

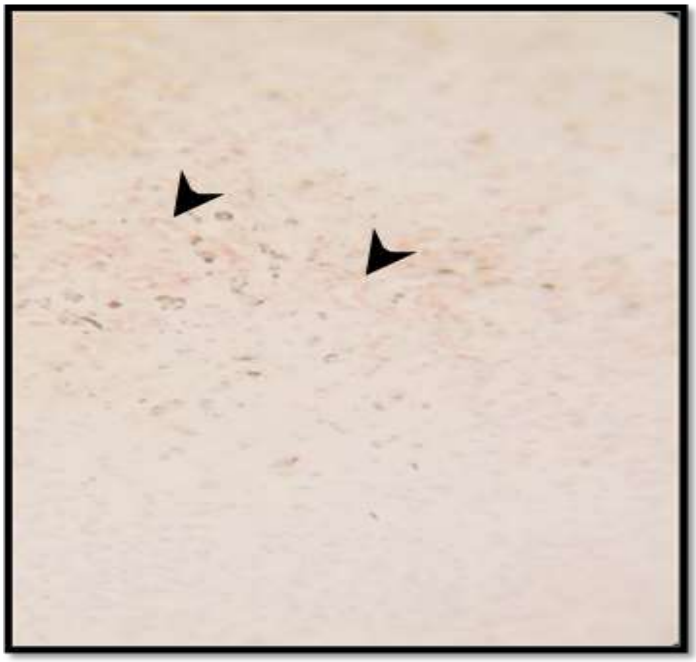

Figure - 11: Skin section of control group at 4th week post wound induction showed formation of new generated blood vessels (black arrow head) H & E (500 X)

Section of skin of stem cells treated group at 4th week reveal marked re-epithelialization in the site of skin wound with normal appearance of epidermal layer as well as appearance of the keratin in the superficial layer. Mature scar in the dermal layer (Figure - 12).

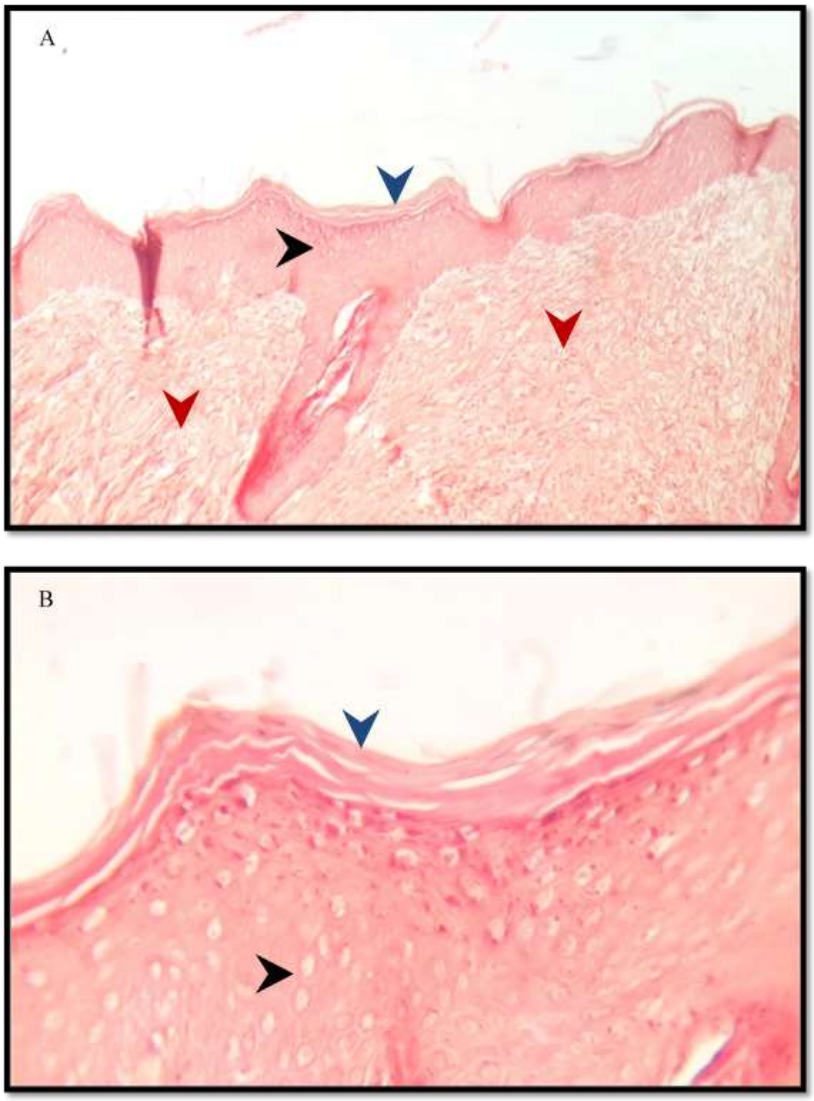

Figure - 12: Skin section of stem cells group at 4th week post treatment showed marked re-epithelization in the site of skin wound (black arrow head) appearance of the keratin in the superficial layer (blue arrow head), mature scar in the dermal layer (red arrow head) H & E A: (125X). B: (500X)

4. Discussion

The results showed advanced reduction in the wound surface area with time in treated groups (T1) compare to the control one (Figures – 3 and 4) and the best result was showed in the stem cells group (T1). The results of different percentages of wound contraction in (Table - 1) showed higher mean percentage of wound contraction in group treated with stem cells (T1) (82.75 %) compare to control group (41 %). Full-thickness skin wounds may result in extensive damage to the different skin structures and the underlying tissues. This damage compromises the homeostatic mechanisms involved in spontaneous healing and therefore clinical intervention is often needed. The primary aim of clinical skin wound treatment is to promote rapid wound repair with functional and aesthetical satisfactory scar tissue formation (Singer and Clark, 1999).

Optimum healing of a cutaneous wound requires a well-orchestrated integration of the complex biological and molecular events of cell migration and proliferation, Extracellular matrix (ECM) deposition, angiogenesis and remodeling (Martin, 1997; Falanga 2005).Regenerative medicine involves the use of living cells to repair, replace, or restore normal function to damaged or defective tissues and organs (Chien, 2008; Polak, 2010). A new type of adult stem cells within fat tissue was identified at the end of the 20th century, when several plastic surgeons first discovered the existence of multilineage stromal cells within fat tissue. Since their discovery, these cells have been referred to by several different names such as Adipose-derived stem cells, Adipose-derived stromal cells (ADSCs), and Adipose stem cells (ASCs). The discovery of ASCs, imparts regenerative medicine with the potential to overcome critical impediments to actual clinical cell therapy applications. ASCs not only have characteristics similar to those of adult stem cells, but also possess two distinct advantages over Bone marrow stem cells (BMSCs). Specifically, ASCs are easily harvested by liposuction under local anesthesia without leaving a conspicuous scar and repeated harvesting, if necessary, is not problematic. Additionally, a large number of cells can be acquired from any type of fat tissue from the body. Therefore, cell culturing may not be necessary to acquire a therapeutic amount of cells. Owing to these two advantages, stem cell therapy can be applied under several limited clinical situations (Jeong *et al*., 2000; Tholpady *et al*., 2006; Lee *et al*., 2012).

Stem cells are viewed as promising candidates for use in cell-based therapies, owing to their capacity for self-renewal and differentiation into diverse mature progeny (Lengner, 2010). Stem cells are characterized by their multipotency and capacity for self-renewal (Behr *et al*., 2010). Their therapeutic potential is largely due to their ability to secrete pro-regenerative cytokines, making them an attractive option for the treatment of different wound (Garg, 2014). Like bone marrow, adipose tissue is derived from the mesenchyme and contains a supportive stroma that is easily isolated. ADSC may be an alternative and less-invasive source of mesenchymal stem cells existing within human adipose tissue (Katz *et al*., 2005; Dicker *et al*., 2005).

Adipose tissue stem cells (ASCs), known as multipotent stem cells, are most commonly used in the clinical applications in recent years. Adipose tissues (AT) have the advantage in the harvesting, isolation, and expansion of ASCs, especially an abundant amount of stem cells compared to bone marrow (Dinh-Toi *et al*., 2019). Microscopic evaluation in this study showed superiority of stem cells group compaction to the control group. Stem cells group heal faster than other groups. Skin injury initiates mechanisms to limit damage and subsequently induce repair. Both phenomena cover a complex cascade of temporal and spatial events that are required for tissue homeostasis. These events include induction and resolution of inflammation on the one hand, and the formation and remodeling of tissue on the other hand with the goal to achieve complete reconstruction of the wounded area (Clark, 1996).

Adipose-derived MSCs (ASCs) can be differentiated into adipogenic, chondrogenic, myogenic, and osteogenic cell lineages in response to specific stimuli (Zuk*et al*., 2002). Alternatively, ASCs may be immediately administered without in vitro expansion or differentiation in culture. The extraordinarily high cell yield from lipoaspirate (as many as 1×10^7 cells from 300 ml of lipoaspirate with at least 95 % purity), as compared with bone marrow aspiration, makes ASCs a particularly attractive cell source for the acute wound setting (Boquest *et al*., 2006).

ASCs have been tested in multiple preclinical trials on wound healing and have been found to significantly enhance cutaneous wound healing and increase blood vessel formation (Teng *et al*., 2014). The acquisition of adipose tissue is much less expensive than bone marrow,

with less invasive operation and available in greater quantities. Clinically relevant stem cell numbers can be extracted from isolated adipose tissue since it possesses higher stem cell proliferation rate than BMSCs. Therefore, adipose tissue represents an abundant, practical, and appealing source of donor tissue for autologous cell replacement (Cowan *et al*., 2004). *In vitro*, ASCs can be differentiated into osteoblasts, chondroblasts, adipocytes, myocytes, and cardiomyocytes in suitable conditions. Adipose tissue consists of 100 - 500 folds higher number of stem cells compared to bone marrow, which makes ASCs an attractive source for human usage. ASCs show therapeutic impacts on angiogenesis, wound healing, and the immune regulatory system (Chu *et al*., 2016; Rogne *et al*., 2018).

In general, ASCs can be isolated from the collected adipose tissues in patients and directly injected into the wounds, bloodstream, or encapsulated in biomaterials and implanted in the wounds. Many investigations showed ASCs can increase the healing rate and decrease healing time both *in vitro* and *in vivo*. ASCs can directly differentiate into specific cell lineages such as keratinocytes, fibroblast-like cells, and endothelial cells, together with the release of growth factors and cytokines, all that promote angiogenesis, development, migration of fibroblasts, and production of fibronectin and collagen (Hassan *et al*., 2014; Si *et al*., 2019).

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